Summary of Ph.D. thesis

EVALUATION OF THE EFFECT OF ANTIVIRAL AND ANTI-INFLAMMATORY COMPOUNDS AGAINST RABIES IN CELL CULTURE AND MOUSE MODEL

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Budapest, 2020

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Introduction and aims

Rabies is a viral, zoonotic disease that is present worldwide. It is transmitted via infected saliva of rabid animals, through their bites. Rabies virus (RABV; *Rhabdoviridae, Lyssavirus*) causes almost 100% lethal encephalomyelitis in any mammalian species including humans.

There are three independent epidemiological cycles of rabies. Urban rabies is maintained by free-roaming community dogs at settlements, which are the main sources of human infections. More than 99% of the 50,000-150,000 annual human casualties of rabies are dog-mediated. Urban rabies is present in most of the African and Asian countries; the highest case number is registered in India. The global eradication of human rabies is an essential issue. To achieve that, in addition to mass dog vaccination and public awareness, the public health system and laboratory diagnostic background has to be developed in endemic regions.

Various wild carnivore species (including foxes, wolves, raccoons, jackals) are the reservoirs of sylvatic rabies, which occasionally infect dogs, cats or humans. Oral vaccination campaigns in Western/Central Europe

and North America were successful in controlling this epidemiological cycle, although it is still widespread in Eastern Europe and Asia. Bat rabies is the third type of rabies that circulates is large bat colonies. In the Americas bat rabies is caused by RABV, whereas in other continents by other *Lyssavirus* genotypes but not RABV.

There are efficient vaccines available against rabies for preventive or post-exposure vaccination (PrEP or PEP) of humans, and for the immunization of animals. The widely used inactivated vaccines provide protection against the development of a clinical disease, even if administered shortly after exposure to the virus in multiple doses: the innate immune system and the rapid induction of an antibody response prevent the virus entry into the central nervous system (CNS). Local infiltration with human rabies immunoglobulins (HRIG) is highly recommended to facilitate vaccination and thus enhance protection. Nonetheless, once the infection of the CNS is established, inactivated vaccines are no longer able to prevent the onset of clinical signs and the subsequent lethal outcome. In addition, no therapeutic option is available to date that could provide significant survival chances in case of rabies with overt clinical signs.

The aim of our international collaborative research program was to find and analyze compounds and combination therapies that can be effectively used in the treatment of rabies encephalitis. The therapeutics involved in our study were chosen based on novel scientific information and experimental results about the pathogenesis of rabies in cellular level.

In the first phase of our studies the effect of compounds with proven or presumed inhibitory effect on RABV replication was evaluated in cell culture. An important goal was to investigate the antiviral effect of combinations of compounds in addition to single compounds alone.

In the second phase the aim was to study the effect of various combination therapies on the survival of rabies-infected mice. In our *in vivo* experiments we made effort to avoid mortality of mice via alleviation of detrimental host responses to infection, using compounds that mitigate the excessive inflammation in the CNS. We planned to establish the most effective treatment regimen possible with the combined use of anti-inflammatory and antiviral drugs.

Materials and Methods

In vitro studies

Five antiviral compounds were involved in the *in vitro* phase of our experiments. Recombinant mouse interferon (IFN)- α and -β (belonging to type-I IFNs), the nucleoside analogues ribavirin and favipiravir, as well as the mitogen-activated protein (MAP) kinase inhibitor sorafenib were studied for their inhibitory effect on RABV replication in mouse neuroblastoma (N2a) cell line. Four non-cytotoxic concentrations of each compound were chosen based on a cytotoxicity test that measures extracellular lactate-dehydrogenase activity of cells. Following the evaluation of the effect of single compounds, virus infected N2a cells were treated also with their respective combinations. Each combination included two compounds, in a higher or a lower concentration. In experiments with combinations only IFN-β was used out of the two type-I IFNs.

Antiviral effect was detected using three different methods. In case of any method the cells were first infected with the fixed lab-adapted RABV strain CVS-11, at a multiplicity of infection (MOI) of 0.1. After 60 minutes adsorption the virus-containing cell culturing medium was replaced to fresh medium containing the antiviral compounds at the desired concentration.

Supernatants of the cell cultures were collected 48 hours later for the purposes of FFA and qRT-PCR tests (see below), followed by fixation of the experimental plates with 80% acetone and fluorescent staining to detect the nucleoprotein of RABV. The infection rate of cell cultures (ratio of fluorescent cells) was semi-quantitatively assessed using an inverted fluorescent microscope.

The infectious titre of RABV in the removed culture supernatants was determined using a fluorescent focus assay (FFA): samples were titrated 10-fold in quadruplicates on N2a cells, then the titration plates were fixed and fluorescent stained after 48 hours. Results were calculated using the Spearman-Kärber method, and expressed in $TCID_{50}$/ml. The same supernatant samples underwent a chromatographic nucleic acid extraction, and then RNA copy numbers of RABV were measured with quantitative reverse transcription (qRT-) PCR. $TCID_{50}$-equivalent values were calculated from raw results in order to allow better comparison with FFA results.

Following current European Union legislation all experimental phases concerning possibly live virus were conducted in biological safety level (BSL)-3 laboratory.

In vivo studies

In vivo experiments were conducted on mouse models: 6-weeks-old female, specified pathogen free mice of the inbred C57Bl/6 strain were housed in a BSL-3 rodent facility. In order to ease the unnecessary suffering of mice, humane endpoint was determined (severe spasms affecting the whole body). Animals that reached endpoint were euthanized. The humane endpoint is equivalent with score 3 of the clinical scoring system we adapted for the study that differentiates 5 scores of rabies clinical course.

Three different mouse experiments were performed. Mice were infected with the wild-type SHBRV-18 RABV strain of bat origin: the virus suspension was inoculated intramuscularly into the left hind leg, under isoflurane anesthesia. Clinical status and body weight were measured twice a day. Animals were enrolled to different experimental groups using a randomizer software: besides the infected-treated groups, untreated virus control and uninfected drug control groups were created. Phosphate-buffered saline (PBS) was administered instead of therapeutics in virus control groups, and instead of virus suspension in the drug control groups.

In the first experiment the challenge virus dose was adjusted to LD_{50}. Mice were treated intraperitoneally (ip.)

daily for 8 days with a combination of the anti-inflammatory compounds sorafenib (MAP-kinase inhibitor), infliximab (antibody against tumor-necrosis factor [TNF]-α) and Ac-YVAD-cmk (caspase-1 [CASP-1] inhibitory peptide). In one group treatment was initiated on the day of infection (4 hours before; -4h group); in other groups 2- or 4-days post infection (pi.), respectively (48h and 96h groups). The overall number of mice was n=70.

In the second experiment virus dose was raised to LD_{100}. The therapeutic combination of the first experiment was supplemented with human rabies immunoglobulin (HRIG). The timing of treatments was following the scheme of the first experiment, except for the length of therapy that was prolonged to 10 days. To elucidate the exact role of HRIG in protection an additional control group of HRIG monotherapy was established. The overall number of animals was n=96.

In the third experiment (n=39) the combination was further supplemented with antivirals from the *in vitro* studies with proven anti-RABV efficiency (type-I IFNs, ribavirin, favipiravir). Administration of the compounds was initiated 4 days (96h) pi. in every case. 30 minutes after the daily treatments 25% mannitol was injected ip. to temporarily open the blood-brain barrier.

Mice reaching humane endpoints and all surviving mice at the end of experiments (day 28 pi.) were terminated, and samples were collected from the brain, spinal cord and parenchymal organs for virologic and immunohistochemistric (IHC) evaluation.

The quantity of SHBRV-18-specific viral RNA in the brain and spinal cord samples of mice was determined using a SyBR Green qRT-PCR method. The presence of nucleoprotein antigens of RABV was also detected, with IHC investigation. In addition, the prepared histological slides were stained with hematoxylin and eosin to reveal signs of inflammation due to RABV replication in the CNS.

At the end of the first experiment blood samples were collected from all surviving mice for a serological assay: fluorescent antibody virus neutralization test was performed to measure the anti-rabies neutralizing antibody level in the sera of animals.

Results

In vitro studies

According to our results, all tested antivirals inhibit the replication of RABV in N2a cells, and the inhibitory effect is concentration-dependent. Regarding the two type-I IFNs, the antiviral potential of IFN-β highly exceeded that

of IFN-α; furthermore, it also outmatched the effect of the 1:1 mixture of the two IFNs. Based on FFA results, at the highest tested concentration (10 IU/ml) IFN-β reduced viral titres by 5.75 log_{10} compared to the untreated virus control, while IFN-α caused a 3.5 log_{10} reduction. At the lowest concentration (0,01 IU/ml) the inhibitory effect of IFNs was only marginal.

Ribavirin and favipiravir provided similar results, although at higher concentrations (10 and 1 μg/ml) ribavirin's antiviral activity was more pronounced than favipiravir's. The two nucleoside analogues reduced RABV titres significantly less than IFN-β; their effect was broadly comparable to IFN-α.

Sorafenib turned out to be an extremely efficient anti-rabies compound: it caused 5.75 and 5.37 log_{10} drop of titres at the two higher concentrations (50 and 5 μM), respectively. The former is identical with the maximal inhibitory effect of IFN-β. Nevertheless, the two lower concentrations (0.5 and 0.05 μM) inhibited RABV replication to a much lesser extent.

Regarding combinations, it was IFN-β that, if combined with any other tested compound, had a more pronounced inhibitory effect on virus replication than the same antivirals as single compounds. However, the

interaction was not synergistic in either case. The most notable effect was achieved by the combination of IFN-β and sorafenib. The combination containing 1 IU/ml IFN-β and 5 μM sorafenib caused greater decrease in viral titres than the single compounds at even 10 times higher concentration; its (logarithmic) relative inhibition approached 80% (77.19%). That value was 48.07% and 68.90% in case of 1 IU/ml IFN-β and 5 μM sorafenib alone, respectively.

The combination of IFN-β with ribavirin or favipiravir also caused an enhanced effect compared to the components of the mixtures, whilst this enhancement is surprisingly influenced by the concentration of ribavirin and favipiravir, instead of IFN-β (which was more effective as a single compound than the nucleoside analogues).

Strong antagonism was shown by the ribavirin + favipiravir, ribavirin + sorafenib and favipiravir + sorafenib combinations. Not only was their combined inhibitory effect below those of the single compounds at the same doses, in certain cases the combinations failed to reduce viral replication at all, even compared to the virus control.

Similar trends to the above-detailed FFA results were revealed by qRT-PCR and the semiquantitative assessment of infection rate of cells. However, the exact

results differed, due to the different nature of the methods (they provide information about different stages of virus replication). The reliability of PCR at very high, as well as that of the infection rate assay at very low viral concentrations fell short of the FFA method. Titres calculated from qRT-PCR copy numbers slightly exceeded the results of the FFA test (except for the highest virus doses).

In vivo studies – 1st experiment

In the first animal experiment the therapeutic combination including inhibitors of MAP-kinases, TNF-α and CASP-1 had no toxic effect on mice; members of the drug control groups remained healthy throughout the experiment with no decrease of body weight. In virus-infected groups, mice that developed clinical signs of rabies lost weight rapidly due to the lack of food intake, dehydration and severe spams.

The first signs of rabies appeared on the 6th day after infection, first in the virus control group, then from the following day on, in the infected-treated groups as well. The disease started with the twitching or paralysis of the inoculated left hind leg, which was followed by progressive spasms usually within half a day. Once the

spams expanded to affect the whole body with high intensity (humane endpoint), mice were humanely terminated.

The survival rate of the virus control group (6/13 mice) was consistent with the LD_{50} dose of the challenge virus. Higher survival rate was observed in the treated groups: there were 8 survivals in the pre-exposure (-4h) group, 9 in the 48h group, and 10 in the 96h group. There were two major waves of mortalities: first between the 7th and 9th day pi., then later around the 12th-13th day.

High amounts of viral RNA were detected with qRT-PCR in the central nervous system of mice euthanized due to rabies, regardless of therapy received. Four surviving animals had positive PCR result for both the brain and spinal cord, and one more for the spinal cord only. However, RNA copy numbers in surviving mice were much lower than in those reaching humane endpoint.

In the spinal cord of surviving mice with a positive PCR result, the nucleoprotein of RABV was also detected, but their brain was negative by IHC test (with one exception). Infected neurons were present in a high number in the CNS of euthanized mice (especially in the spinal cord, brainstem, cerebellum and cerebrum), accompanied with histopathological signs of inflammation

(mononuclear cell infiltration). Concerning all PCR-positive animals, we compared antigen levels determined in the brain and spinal cord between groups with different therapeutic schedules. We found a pattern similar to surviving rates: later start of treatment resulted in a lower level of infection in the CNS.

Seroconversion of all surviving RABV-infected mice was demonstrated using virus neutralization test on serum samples. Their average antibody titer (0.61 IU/ml) exceeded the level providing a certain protection (0.5 IU/ml), according to the World Health Organization (WHO). Surviving animals with positive PCR-/IHK results had higher antibody level than the average: 1.09 IU/ml.

The clinical signs of one mouse belonging to the 48h treatment group appeared later than in other mice (on the 16th day pi.), and its clinical course of rabies was stopped in an early stage (paralysis of the left hind leg). The mouse survived until the end of the experiment; furthermore, it started to gain body weight in the last couple of days, despite showing severe weight loss previously due to rabies. RABV-specific RNA and antigens were detected both in the brain and spinal cord, and it had a higher than average level of neutralizing antibodies (0,87 IU/ml).

In vivo studies – 2nd experiment

In the second experiment, the therapeutic combination including HRIG in addition to the anti-inflammatory compounds used in the first experiment showed no toxic effect. The clinical course and body weight changes were similar to those observed in the first experiment.

Due to the elevation of the dose of the challenge virus to LD_{100}, mortality in the virus control group was close to 100% (3/26 surviving mice). The combination therapy had a significant protective effect: survival rates were 12/13 in the -4h treatment group, 7/13 in the 48h group, whereas 6/13 in the 96h group. 10 mice out of 13 survived infection in the extra control group receiving HRIG monotherapy instead of the full combination (following the -4h scheme).

Regarding viral RNA levels only the results of the -4h group was significantly lower compared to the virus control group. Only one surviving animal belonging to the virus control group provided positive PCR results for brain and spinal cord samples.

In vivo studies – 3rd experiment

Complementation of the combination used in the second experiment with antiviral compounds found to be effective in our *in vitro* studies failed to enhance survival of rabies.

The weight loss observed in the drug control group starting from the day of initiation of treatment, and then subsequent mortality exceeding the 50% rate along with aspecific clinical signs undoubtably indicate toxicity of the tested combination. Two members of the infected-treated group also showed toxicity-related signs before death, and the 100% mortality of this group exceeded that of the virus control group (84.6%).

High levels of viral RNA and antigens were found in the CNS of mice terminated due to rabies, regardless of experimental group. The PCR and IHC results of the two surviving mice of the virus control group were positive, while RABV-specific RNA but not antigens were detected in the two animals of the infected-treated group that died due to toxicity.

Discussion

In vitro studies

RABV interferes with immune functions through multiple mechanisms, among which one of the most relevant is that is blocks the synthesis of type-I IFN. Despite the suppression of the endogenous IFN production the virus is still responsive to exogenous IFNs, which was shown by the results of our experiments on N2a cell line. We

demonstrated that the inhibitory potential of IFN-β on RABV multiplication highly surpasses that of IFN-α. According to our results, the combination of the two type-I IFNs does not potentiate the antiviral effect since both of them bind to the same cell surface receptor (IFNAR-1/-2).

The *in vitro* activity of ribavirin against RABV is known from former studies. Our results also confirmed this finding. The inhibitory effect of another nucleoside analogue, favipiravir, turned out to be lower than ribavirin's; in contrast to the findings of other research groups testing the compound against a variety of other RNA viruses. Favipiravir is converted to its active metabolite in the cells via phosphoribosylation, but the efficiency of this metabolic process is different in various cell types. It is possible that the use of other cell lines or the investigation of the active form of the molecule could lead to an increase of the moderate effect we observed.

Despite being basically an antitumor agent, sorafenib impedes the replication of multiple viruses. We demonstrated that sorafenib significantly reduces the replication of RABV; its antiviral effect was the highest among all tested compounds. Sorafenib inhibits numerous MAP-kinase cascades, albeit having the greatest impact on Raf/MEK/ERK signaling. This pathway

plays an important role in the cellular pathogenesis of rabies; its inhibition affects both the synthesis of viral proteins and viral nucleic acid replication.

In most research about antiviral treatments against rabies, only single compounds are investigated. An important goal of our study was to analyze the effect of combinations of compounds inhibiting RABV replication. We demonstrated that the combination of IFN-β with sorafenib, ribavirin or favipiravir enhances the inhibitory effect, though not is a synergistic manner. The IFN-β + sorafenib combination was particularly effective, but further research is required for the understanding of the mechanism of the combined effect.

The significant antiviral activity of the combination of type-I IFNs with ribavirin or favipiravir against RNA viruses is already described. However, the increase in the combined effect compared to the individual components was different. According to our studies, the anti-RABV effect of IFN-β can be slightly enhanced with the addition of ribavirin or favipiravir, where the effect is primarily limited by the concentration of the nucleoside analogue.

The ribavirin + favipiravir combination exhibits a pronounced synergy against heamorrhagic fever viruses, but in case of other viruses (e.g. Zika virus) an

antagonistic effect was reported. We also revealed antagonistic interaction of the combination against RABV. The mechanisms of action of the two compounds overlap in certain aspects, which can explain antagonism. However, another effect of ribavirin (depletion of intracellular GTP levels through inhibition of the IMPDH enzyme) may increase the incorporation of favipiravir into viral RNA copies, facilitating its inhibitory effect. It is plausible that the emphasis of the different processes exerted by ribavirin is different against various viruses, which can promote a synergistic or an antagonistic effect of its combination with favipiravir.

The combination of sorafenib with ribavirin or favipiravir showed strong antagonism; presumably some of the MAP kinases inhibited by sorafenib are important for the metabolism or effect of the nucleoside analogues.

In vivo studies

The aim of our *in vivo* studies was to reduce detrimental host response of the immune system to rabies, using combinations of immunomodulatory compounds.

In the first experiment mice infected with the SHBRV-18 strain of RABV were treated with inhibitors of MAP kinases, TNF-α, and CASP-1. The combination therapy

reduced mortality by 15-30%, depending on the timing of treatment. Due to the relatively low group sizes the difference between treated groups and the virus control is only significant if the survival rate of the virus control group that approximately fit to LD_{50} is considered as a reference value (exact binomial test).

Interestingly, higher survival rates were observed at groups with later start of the combination therapy. There was a similar trend regarding the amounts of RABV antigens in the CNS: the highest antigen level was detected in the -4h treatment group, while the lowest was found in the 96h group. This can be explained by the fact that the anti-inflammatory agents were administered for 8 days, therefore therapy with earlier initiation also finished earlier. Longer maintained treatment, however, could possibly prevent late mortalities around days 12-13 pi.

The RNA of RABV was detected by PCR in the brain and spinal cord of four surviving mice, and only in the spinal cord of one mouse. Viral antigens were also detectable in the spinal cord of these animals; however, IHC assay of brain samples provided positive result only for one mouse that survived rabies despite developing early clinical signs. Wild-type RABV strains are known to delay immune response through various immune evasion

mechanisms, but the combination therapy likely provided extra time for the delayed entry of lymphocytes into the CNS by alleviating inflammatory processes. Infiltrating B cells are capable of clearing RABV infection in the brain via local production of antibodies. In PCR-/IHC-positive surviving mice the immune system could control the spread of infection with the help of the anti-inflammatory therapy before the appearance of clinical signs or even shortly thereafter.

There are two major phases of inflammation involved in rabies pathogenesis: the early stage can facilitate survival but is only triggered by infection of attenuated RABV strains. It is the initial reaction of the innate immune system at the periphery followed by a rapid induction of adaptive immunity. The chemoattractive environment, IFN effects, dendritic cell action and antibody production all contribute to the prevention of an overwhelming CNS invasion of the virus. Wild-type strains instead induce a later, destructive wave of inflammation that takes places during the mass viral replication in the CNS. Microglia cells are key participants of this late cascade: they produce nitric oxide and TNF-α that are harmful in the neuronal tissue. From the signaling pathways associated with programmed cell death, CASP-1-mediated

pyroptosis damages neurons, while apoptosis triggered via the FasL/Fas mechanism causes a significant loss of invading immune cells.

Sorafenib intervenes in the Raf/MEK/ERK pathway, reducing the induction of nitric oxide, TNF-α and FasL. Infliximab is an antibody against TNF-α, whereas Ac-YVAD-cmk inhibits CASP-1 and thus pyroptosis. Combination of the three compounds could therefore mitigate the late inflammatory phase resulted in an increased survival chance in mice infected with the wild-type SHBRV-18 virus strain. This is consistent with our finding that the longer maintained treatment provides higher survival rates and reduced viral load in the CNS.

In the second experiment the addition of HRIG to the combination almost completely prevented mortality in case of a prompt start of treatment (-4h group). The early presence of high number of antibodies could lead to the neutralization of RABV still at the periphery, preventing or reducing viral entry to the CNS. Nevertheless, when mice were treated with HRIG only, lower survival was observed, which means that the immunomodulatory compounds also contribute to the protection. However, in case of a later start of therapy the beneficial effect of HRIG is moderate as the penetration of peripheral antibodies

into the brain is limited at wild-type RABV infection. The lower but still significant increase in survival rates could therefore be dominated mainly by MAP kinase, TNF-α and CASP-1 inhibitors rather than HRIG.

In the third experiment the combination was supplemented with IFNs, ribavirin and favipiravir, based on their *in vitro* efficiency. In order to increase the entry of therapeutics, immune cells and peripheral antibodies to the CNS, mannitol treatment was only applied to temporarily open the blood-brain barrier. In this experiment, protective effect of the treatment could not be demonstrated, since the combination had toxic effects on mice. As the individual components are non-toxic at the concentrations used, there may have been interactions between certain drugs or excipients. Further research is needed to elucidate the details of this toxic reaction.

Our animal experiments demonstrated that the inhibition of the late inflammatory response to RABV infection can promote survival chances. It is recommended to maintain therapy for a longer time to provide time for the adaptive immunity to clear CNS infection. Early administration of antibodies can further facilitate protection. Our studies provide valuable data about therapeutic intervention in rabies.

New scientific results

1) The substantial *in vitro* antiviral effect of the multikinase inhibitor sorafenib against rabies virus was first proven.

2) The *in vitro* anti-rabies effect of the combinations of interferons with other antiviral compounds were first analyzed and thus is was revealed that the combination of IFN-β with sorafenib, ribavirin or favipiravir greatly inhibit the replication of rabies virus.

3) It was shown that the use of any pairwise combination of ribavirin, favipiravir and sorafenib against rabies results in strong antagonism and is therefore not recommended.

4) It was demonstrated that the combination of inhibitors of certain pro-inflammatory cascades and cytokines (CASP-1, MAP-kinase and TNF-α inhibitors) enhance survival chances in rabies-infected mice, presumably by inhibition of the late inflammatory response in the central nervous system.

5) It was demonstrated that the combination of CASP-1, MAP-kinase and TNF-α inhibitor compounds with anti-rabies antibodies greatly enhance survival of rabies in mouse model and that this beneficial effect is more pronounced than that the antibodies alone can provide.

Peer-reviewed publications related to the thesis

Marosi A.: **Új ismeretek a veszettség kórfejlődéséről és immunológiájáról**. Magyar Állatorv. Lapja, 141. 607-622, 2019.

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Acknowledgements

First of all, I have express my gratitude to my supervisor, Tamás Bakonyi, who led me into the world of virology research and entrusted me with this exciting but also challenging research field.

Special thanks to all members of our Virology Research Group: Petra Forgách, Győző Bakonyi, Gyöngyi Hofbauer and Magdi Kaposi. I am also grateful to Miklós Rusvai, László Fodor, Dóra Gerényi, Nikoletta Bíró and to all the former or current colleagues at the Department of Microbiology and Infectious Diseases who provided any kind of help in my work.

I owe many thanks to Barbara Forró, Orsolya Felde, Alexandra Pásztor, Kinga Görföl-Sulyok, Zsuzsa Kreizinger, Miklós Gyuranecz along with others from the Institute for Veterinary Medical Research. I am also thankful to Károly Erdélyi and Ákos Hornyák. I think with gratitude of members of the ASKLEPIOS consortium; among them I have to highlight Karen Mansfield, Anthony Fooks, Ashley Banyard, Penelope Koraka, Lucie Dufkova, Daniel Ruzek and Dirk Jochmans.

Finally, I am grateful for the patience of my loved family; they are worth working and living for.